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AN ALKALINE PROTEASE:-SUBTILISIN CARLSBERG BY RECOMBINANT DNA TECHNIQUES

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(56) Prior Art Documents
US 3790482
US 3557002

(57) Claim

1. Isolated, double-stranded deoxyribonucleic acids consisting of the following structural gene which only codes for a polypeptide:

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   10          20          30          40
...GCGCAAA CCGTTCCTTA CGGCATTCCT CTCATTAAAG
CGGACAAAGT GCAGGCTCAA GGCTTTAAGG GAGCGAATGT
AAAAGTAGCC GTCCTGGATA CAGGAATCCA AGCTTCTCAT
CCGGACTTGA ACGTAGTCGG CGGAGGAAGC TTTGTGGCTC
GCGAAGCTTA TAACACCGAC GGCAACGGAC ACGGCACACA
TGTTGCCGGT ACAGTAGCTG CGCTTGACAA TACAACGGGT
GTATTAGGCG TTGCGCCAAG CGTATCCTTG TACGCGGTTA
AAGTACTGAA TTCAAGCGGA AGCGGATCAT ACAGCGGCAT
TGTAAGCGGA ATCGAGTGGG CGACAACAAA CGGCATGGAT
GTTATCAATA TGAGCCTTGG GGGAGCATCA GGCTCGACAG
CGATGAAACA GGCAGTCGAC AATGCATATG CAAGAGGGGT
```

.../2

TGTCGTTGTA GCTGCAGCAG GGAACAGCGG ATCTTCAGGA
AACACGAATA CAATTGGCTA TCCTGCGAAA TACGATTCTG
TCATCGCTGT TGGTGCGGTA GACTCTAACA GCAACAGAGC
TTCATTTTCC AGCGTCGGAG CAGAGCTTGA AGTCATGGCT
CCTGGCGCAG GCGTATACAG CACTTACCCA ACGAACACTT
ATGCAACATT GAACGGAACG TCAATGGCTT CTCCTCATGT
AGCGGGAGCA GCAGCTTTGA TCTTGTCAAA ACATCCGAAC
CTTTCAGCTT CACAAGTCCG CAACCGTCTC TCCAGCACGG
CGACTTATTT GGGAAGCTAA TTCTACTATG GGAAAGGTCT
stop
GATCAATGTC GAAGCTGCCG CTCAA TAA....

- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that it has the following structure:

- a promoter which is recognized by the RNA-polymerase of microorganisms of the genus Bacillus,
- a ribosome binding site,
- a start codon,
- a leader sequence,
- a stop codon,

DNA-sequences containing up to 400 base pairs without any special expression effect optionally being present at both ends.

6. A process for selecting microorganism strains containing hybrid plasmids according to Claims 2 to 5, characterized in that

- in a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and
- the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

2. Hybrid plasmids for microorganisms of the genus Bacillus, characterized in that they contain at least one double-stranded DNA according to Claim 1 in operable form.

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COMPLETE SPECIFICATION

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Complete Specification for the invention entitled:

"AN ALKALINE PROTEASE, A PROCESS FOR THE PREPARATION OF HYBRID VECTORS AND GENETICALLY TRANSFORMED MICROORGANISMS"

The following statement is a full description of this invention, including the best method of performing it known to us

SBR/TGK/182W

D 7143

A B S T R A C T

An alkaline protease, a process for the preparation of hybrid vectors and genetically transformed microorganisms

A structural gene which codes for the protease subtilisin Carlsberg or proteolytically active sub-units or proteolytically active variants thereof, including their leader sequences, is described. Hybrid plasmids and microorganism strains containing this structural gene and also a process for separating these microorganism strains from inactive strains are also described.

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Patent Application

D 7143

An alkaline protease, a process for the preparation of hybrid vectors and genetically transformed microorganisms

This invention relates to a method for transforming microorganisms of the genus Bacillus into producers of the protease subtilisin Carlsberg and variants thereof and, to that end, describes the preparation of double-stranded deoxyribonucleic acids which only code for subtilisin Carlsberg or variants thereof, hybrid vectors which contain these isolated double-stranded deoxyribonucleic acids together with the structural gene of the leader protein, start and stop codons in an operable sequence, a ribosome binding site and promotor, and also host organisms of the genus Bacillus for these hybrid plasmids.

There are several known proteases which are produced by microorganisms, more especially bacteria and fungi, and which are exported from the cell by active transport so that they collect in the culture medium. Numerous enzymes of this type are already being commercially used in processes where proteins have to be degraded. Important applications includs, for example, detergents and cleaning preparations and even animal foods. The proteases are normally clasified according to the pH-range in which they show optimum activity and, accordingly, are known as alkaline, neutral and acidic proteases. The alkaline

proteases are of particular importance in detergents and cleaning preparations. One important group is known by the generic name of subtilisin. This name derives from Bacillus subtilis because inter alia strains of this species are capable of producing subtilisin. In recent years, researchers have succeeded in separating from one another and structurally elucidating various naturally occurring types of subtilisin (E.L. Smith et al, J. Biol. Chem. (1968) 243 (9), 2184. In this connection, it has been found that two types of subtilisin are of particular importance in practice, namely subtilisin BPN' and subtilisin Carlsberg. Subtilisin BPN' is often obtained from Bacillus amyloliquefaciens, whereas subtilisin Carlsberg, for example, may be obtained by cultivation of strains of the species Bacillus licheniformis. Many Bacillus strains often excrete both proteases in admixture with neutral proteases (metalloproteases).

In view of the commercial significance of enzymes, which may be regarded as somewhat greater in the case of the protease subtilisin Carlsberg than in the case of the protease subtilisin BPN', numerous attempts have already been made to find strains which produce one enzyme or the other or mixtures thereof and which are also suitable for commercial preparation of the products. In this connection, reference is made for example to the following German Offenlegungsschrifts or Patent Specifications: DE 19 40 488, DE 20 18 451, DE 20 44 161, DE 21 01 803, DE 21 21 397 and DE 29 25 427, and also to GB 1 263 765, US 3 623 957, US 4 264 738 and EP-A 6 638. The applications or patents mentioned are concerned with classical mutation processes in which the frequent repetition of mutation and selection steps ultimately gives product strains which are optimized in regard to yield and product quality. It is known among experts that a mutation process such as this takes place statistically so that it is hardly

likely to give "tailored" strains which have objectively the highest possible performance level. In addition, cultivated strains such as these may also tend towards back-mutations, i.e. they lose at least some of their favorable properties and become "wild".

A significant advance was provided by the gene recombination technique (Cohen and Boyer, US 4 237 224). This technique enables a foreign gene to be expressed and reproduced in a microorganism by suitably opening a plasmid, connecting it to the foreign double-stranded deoxyribonucleic acid, reclosing it to form the ring and inserting it into a suitable host organism. Applying this basic technique, European Patent Application 0 130 756 describes the isolation of double-stranded deoxyribonucleic acids which code on the one hand for subtilisin BPN' and on the other hand for neutral proteases (metallo-proteases). The insertion of the double-stranded deoxyribonucleic acids into vectors and the modification thereof is also described. Although the gene-technological production and modification of hydrolases (i.e. proteases, other amylases, esterases, etc.) is claimed, it does not seem possible to apply the steps illustrated with reference to the particular example of subtilisin BPN' to other systems without inventive activity. This would appear all the more to be the case since, according to the teaching of the European Patent Applications cited above, the separation of the gene coding for hydrolase from the gene pool of the starting organism is said to take place using a marked oligonucleotide chain, this oligonucleotide chain corresponding to a partial sequence of the hydrolase and being used in its various possibilities - as predetermined by the genetic code. Considerable experimental effort is required of the expert here, in other words several oligonucleotide chains have to be synthesized in appropriate purity. In addition, the method involved presupposes knowledge of

the amino acid sequence of the desired product.

The inventors set themselves the task of finding microorganism strains which are capable of producing subtilisin Carlsberg, the generic name subtilisin Carlsberg also being intended to include in particular a variant of the enzyme which has two modifications. Another task was to provide suitable hybrid plasmids which could be inserted into and were stable in microorganisms of the genus Bacillus and which code for the production and excretion of subtilisin Carlsberg. Finally, the inventors set out to provide operable, isolated, double-stranded deoxyribonucleic acids which code for subtilisin Carlsberg and which contain the nucleotide sequences necessary for the production and excretion of the enzyme. Another task which the inventors set themselves was to use an immunological detection method simplified by comparison with the isolation method described in European Patent Application 0 130 756 by preformed, marked oligonucleotide sequences in the selection of Carlsberg-positive microorganisms.

In a first embodiment, therefore, the present invention relates to isolated, double-stranded deoxyribonucleic acids consisting of

- a structural gene which only codes for a polypeptide,
- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that they have the following structure;

- promoter recognized by the RNA-polymerase of microorganisms of the genus Bacillus,
- ribosome binding site,
- start codon,
- a leader sequence,
- structural gene which codes for subtilisin Carlsberg

or its proteolytically active sub-units or proteolytically active variants, including their leader sequences,

- stop codon,

DNA-sequences containing up to 400 base pairs without any special expression effect optionally being present at both ends.

The isolated, double-stranded deoxyribonucleic acids according to the invention contain the individual sub-sequences in an operable sequence, i.e. they are in functional relationship to one another. This means that a promotor is present which has the recognition sequence for the RNA-polymerase and that it is followed by a binding site for the ribosomes. Then follows a start codon operable in bacillus, preferably the start triplet GTG. The start triplet is followed by the actual signal sequence for the leader protein and then for the matured subtilisin Carlsberg or its variants or sub-units. The leader sequence is understood to be a prosequence, a presequence or a pre-/pro-sequence which is preferably responsible for the excretion of the enzyme from the cell. Finally, the structural sequence is terminated by a stop codon, preferably by a stop triplet recognized by Bacillus, such as for example TAA. This may be followed by a terminator sequence, preferred sequences being those which are capable of pairing in themselves and which thus lead to loop formation (stem loop). The function of terminator sequences such as these is to terminate the synthesis of the messenger-RNA for the particular gene product.

The isolated, double-stranded deoxyribonucleic acids described may be adjoined at both ends by further sequences, preferably those to which no special expression effect can be ascribed.

The sequence for the matured enzyme may be subject

to certain variations within the scope of the invention. In a first embodiment, sequences which code for subtilisin Carlsberg are provided. Preferred sequences are sequences made up solely of codons which are usual in Bacillus. In one preferred embodiment of the invention, isolated deoxyribonucleic acids containing the following sequence for the matured enzyme are claimed:

```
    10         20         30         40
...GCGCAAA CCGTTCCTTA CGGCATTCCT CTCATTAAAG
CGGACAAAGT GCAGGCTCAA GGCTTTAAGG GAGCGAATGT
AAAAGTAGCC GTCCTGGATA CAGGAATCCA AGCTTCTCAT
CCGGACTTGA ACGTAGTCGG CGGAGGAAGC TTTGTGGCTC
GCGAAGCTTA TAACACCGAC GGCAACGGAC ACGGCACACA
TGTTGCCGGT ACAGTAGCTG CGCTTGACAA TACAACGGGT
GTATTAGGCG TTGCGCCAAG CGTATCCTTG TACGCGGTTA
AAGTACTGAA TTCAAGCGGA AGCGGATCAT ACAGCGGCAT
TGTAAGCGGA ATCGAGTGGG CGACAACAAA CGGCATGGAT
GTTATCAATA TGAGCCTTGG GGGAGCATCA GGCTCGACAG
CGATGAAACA GGCAGTCGAC AATGCATATG CAAGAGGGGT
TGTCGTTGTA GCTGCAGCAG GGAACAGCGG ATCTTCAGGA
AACACGAATA CAATTGGCTA TCCTGCGAAA TACGATTCTG
TCATCGCTGT TGGTGCGGTA GACTCTAACA GCAACAGAGC
TTCATTTTCC AGCGTCGGAG CAGAGCTTGA AGTCATGGCT
CCTGGCGCAG GCGTATACAG CACTTACCCA ACGAACACTT
ATGCAACATT GAACGGAACG TCAATGGCTT CTCCTCATGT
AGCGGGAGCA GCAGCTTTGA TCTTGTCAAA ACATCCGAAC
CTTTCAGCTT CACAAGTCCG CAACCGTCTC TCCAGCACGG
CGACTTATTT GGGAAGCTAA TTCTACTATG GGAAAGGTCT
                                stop
GATCAATGTC GAAGCTGCCG CTCAA TAA....
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This sequence codes for a matured subtilisin Carlsberg which deviates in positions 158 and 161 in the aminoacid sequence.

In another embodiment of the invention, the claimed

isolated, double-stranded deoxyribonucleic acids code for non-modified subtilisin Carlsberg, i.e. they contain the amino acid asparagine instead of the amino acid serine in position 161 and the amino acid serine instead of the amino acid asparagine in position 158. It follows from this that the base triplets (genetic codes) usual in Bacillus for these amino acids must be present in the corresponding isolated deoxyribonucleic acids.

The isolated, double-stranded deoxyribonucleic acids contain a sequence for a leader protein between the start triplet and the sequence coding for the matured enzyme.

The present invention also relates to hybrid plasmids for microorganisms of the genus Bacillus, characterized in that they contain at least one double-stranded DNA of the invention in operable form. According to the invention, a hybrid plasmid such as this is intended to have the following structure. It should have one resistance marker at its disposal and should comprise at least one enzymatic cleavage site for specific restriction enzymes, as few cleavage sites as possible, i.e. no more than 4 and preferably no more than 1 cleavage site, being present per restriction enzyme. In other words the hybrid plasmids are intended to be able to be linearized by the use of special enzymes.

The invention further provides a process for selecting microorganism strains containing hybrid plasmids of the invention, characterized in that

- in a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and
- the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which

are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

The invention also provides microorganism strains of the genus Bacillus containing hybrid plasmids of the invention.

The invention provides a process for the preparation of subtilisin Carlsberg, characterized in that microorganism strains of the invention are cultivated.

The function of the resistance marker is to enable microorganisms containing the hybrid plasmids, which have been transformed in this way, to be distinguished by their resistance from those which do not contain these hybrid plasmids. Various starting plasmids are available to the expert in this field. They have resistance markers against, for example, tetracycline, kanamycin, chloramphenicol or other antibiotics. Among the various possible starting plasmids for preparing the hybrid plasmids, those which are suitable for microorganisms of the genus Bacillus should

be selected. Accordingly, the hybrid plasmids according to the invention are best obtained from starting plasmids which in turn are suitable for bacillus. Various starting plasmids of this type have been described in the specialist literature. A large number of suitable plasmids can be ordered from depositories, for example from the Bacillus Genetic Stock Center. Suitable starting plasmids are, for example the following: pBCE16, pC194, pUB110, pE194, pSA2100, pPL608, pBD64. References to these plasmids can be found in the following literature:

Bernhard K., Schrempf H., Goebel W.,
1978, J. Bacteriol. 133: 897 - 903

Gryczan, T.J., Contente, S., and Dubnau, D.
1978, J. Bacteriol. 134: 318 - 329

Williams, D.M., Duvall, E.J. and Lovett, P.S.,
1981, J. Bacteriol. 146: 1162 - 1165

Cryczan, T., Shivakumar, A.G., and Dubnau, D.,
1980, J. Bacteriol. 141: 246 - 253

Ehlich, S.D., Bursztyn-Pettegrew. H., Stroynowski, J., and Leaderberg. J., 1977 in Recombinant Molecules: Impact on Science and Society, pp. 69 - 80, Raven Press, New York

Weisblum, B. Graham, M.Y., Cryczan, T., and Dubnau, D.
1979, J. Bacteriol. 137: 635 - 643

When selecting suitable starting plasmids, it is important to remember that they should be able to be linearized by specific restriction enzymes without losing their characteristic properties. The characteristic properties of the starting plasmids are understood to include

their replicatability, their resistance and the possibility for expression in Bacillus. Current specific restriction enzymes with which suitable starting plasmids can be linearized are, for example, Bam HI, Eco RI, Hin DIII and Pst I.

The hybrid plasmids according to the invention contain the double-stranded isolated deoxyribonucleic acids inserted in one or more such cleavage sites. For example, the starting plasmid pBCE16 which is particularly suitable for the purposes of the invention may be linearized with Bam HI and then reclosed to form the ring after insertion of the double-stranded deoxyribonucleic acids according to the invention. Another embodiment of the invention is directed not only to the ring-closed hybrid plasmids, but also to the corresponding ring-opened forms and also adducts of 2, 3 or more ring-opened hybrid plasmids.

The present invention also relates to microorganism strains of the genus Bacillus which contain hybrid plasmids of the type claimed in Claim 2 and the following Claims. For producing or rather transforming suitable microorganism strains, the expert will choose those strains which accommodate hybrid plasmids and in which these hybrid plasmids show adequate stability. Strains of the species Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens or even Bacillus cereus may advantageously be transformed. In this connection, transformed strains may be distinguished from non-transformed strains by detections of the protease formed, for example by the immunological method described hereinafter.

In one embodiment of the invention, the strains transformed are Bacillus strains, for example Bacillus subtilis strains, which for their part are not able to form protease. Strains such as these may be obtained from depositories. It is thus possible to obtain strains which are only capable of producing the desired subtilisin Carlsberg or the

described variant of subtilisin Carlsberg. However, it is also possible similarly to produce strains which thus secrete subtilisin Carlsberg in addition to other proteases or amylases. Specifically, it is thus possible by selection to obtain strains showing particularly high enzyme production.

The double-stranded deoxyribonucleic acids according to the invention are isolated from protease-forming strains of the species Bacillus licheniformis, among which the following strains for example are suitable: Bacillus licheniformis: ATCC 10716, DSM 641 as well as the strains DSM 3406 and DSM 3407 (Laboratory specification "A 441 and "A 453" (sent to DSM for deposition by letter of 19.7.85). These strains are cultivated as described in German Patent 29 25 427. The cells are then harvested. The harvested cells are subjected to protoplasia, i.e. the murein cell wall is disrupted with suitable enzymes, particularly lysozyme, after which the actual cell membrane is disrupted with detergents, such as for example sodium dodecylsulfate. The lysates thus formed may first be subjected to a treatment with RNA-cleaving enzymes (RNase) and/or proteinase K. Then follows the DNA isolation step.

Several methods are known to the expert for isolating chromosomal DNA. They may be carried out either individually or in combination. In one important method, the lysate is first subjected to fractional precipitation in ethanol to enable high molecular weight DNA-fragments to be separated off and thus isolated. Another method is based on separation in a density gradient. To this end, a density gradient is produced in a caesium chloride/DNA-solution. The total quantity is divided up into various fractions so that the DNA collects in certain of these fractions which may then be separated off and further processed. In many cases, it is best to eliminate most of the proteins before or during isolation of the DNA by extraction with a mixture of chloroform and isoamylalcohol.

Basically, the plasmid-DNA may be separated off in

the same way, although in this case it is best to introduce ethidium bromide (a red dye capable of intercalation) into the caesium chloride density gradient, thus artificially establishing a difference in density between plasmid and chromosomal-DNA.

The purified, isolated deoxyribonucleic acids are then subjected to cleavage with restriction enzymes in a suitable buffer (at pH values of from 7 to 7.5). Suitable restriction enzymes are, for example, Bam HI, Sau 3A or other enzymes which are capable of cleaving DNA at specific sites. The chromosomal DNA may be treated with the same enzymes as the plasmid DNA or even with different enzymes. In either case, it is important to ensure that cleavage of the chromosomal DNA is not complete in order thus to obtain fragments of adequate length which with sufficient probability contain the structural gene intact. The isolated, double-stranded deoxyribonucleic acids are mixed with the linear plasmids, the chromosomal deoxyribonucleic acids preferably being used in a large excess by weight over the linearized plasmids. A ratio of from 1:2 to 1:20 is preferably used, a ratio of from 1:5 to 1:10 being particularly preferred. The mixed deoxyribonucleic acids, which should then be present in a concentration of around 0.2 mg per ml, are ligated with DNA-ligase in a following step.

The ligation mixture thus obtained is used to transform competent cells of Bacillus species, such as Bacillus subtilis or Bacillus licheniformis. If it is desired to recognize successfully transformed strains from their protease formation, it is preferred to transform those strains which, for their part, are not capable of forming protease or at least are not capable of forming subtilisin Carlsberg. However, the starting strains may also be transformed. In order to obtain competent cells, the strain intended for transformation is cultivated to the stationary phase in a first

minimal medium and is then further cultivated in a second, dilute minimal medium. The hybrid plasmid is then offered as such to the microorganisms thus pretreated, generally in buffer solution. Thereafter a growth phase may be carried out in a full medium (literature Cahn, F.H. and Fox, M.S. (1968) J. Bacteriol. 95: 867 - 875). The microorganisms thus obtained are then subjected to a selection process. In this process, they are normally first cultivated in a medium containing an antibiotic against which the hybrid plasmid imparts resistance. In this way, organisms actually containing the hybrid plasmid in one or more copies may be obtained in a first selection step. The microorganisms are then selected for their ability to form subtilisin Carlsberg in another selection step.

An immunological method may advantageously be used for this selection step. A preferred method is based on the work of Broome and Gilbert. In this method, antibodies against subtilisin Carlsberg are first obtained by immunization of rabbits, as described by Buckel. Some of the antibodies are enriched by precipitation with ammonium sulfate and DEAE-chromatography whilst the rest are additionally enriched by affinity chromatography with activated Sepharose 4B.

Proof of the existence of subtilisin Carlsberg may be provided as follows:

A PVC film is coated with antibodies against subtilisin Carlsberg. The coated film is placed on the bacterial cultures to be investigated. Any subtilisin Carlsberg present is bound by the antibodies. In another step, the subtilisin Carlsberg antibodies purified by affinity chromatography, onto which peroxidase has been coupled in a previos step, are bound to the antibody-subtilisin Carlsberg complex.

(1) Broome, S. & Gilbert, W. (1978) Proc. Natl. Acad. Sci. USA 75, 2746 - 2749

(2) Buckel, P & Zehelein, E. (1981) Gene 16, 149 - 159

The detection is carried out with tetramethylbenzidine as the peroxidase substrate. In the presence of subtilisin Carlsberg-positive clones, a blue-green coloration develops.

The immunological separation method described above affords considerable advantages in connection with the present invention. Thus, it enables strains which actually form subtilisin Carlsberg to be specifically recognized. This is particularly important in cases where the strains used also comprise other metabolism products which do not respond to the immunological method. Another advantage of this method of detection is that it enables the clones to be safely isolated from a large number of transformed and non-transformed strains.

From the clones thus isolated which contain the hybrid plasmids according to the invention, the hybrid plasmids may be isolated as described at the beginning by cell lysis and subsequent separation in the density gradient in the presence of ethidium bromide. Purified hybrid plasmids are made available in this way. Troublesome ethidium bromide may be separated off by extraction with isopropanol. The purified hybrid plasmids may be inserted into other Bacillus strains under the conditions described above.

In another embodiment of the invention, the transformation may be carried out using protoplasts rather than competent cells. To this end, protoplasts are initially prepared by disrupting the murein cell wall of microorganisms of the genus Bacillus with suitable enzymes and then allowing the hybrid plasmids to act together with polyethylene glycol on the protoplasts, preferably working in buffer solutions. The protoplasts are then further cultivated on a regeneration medium and the intact microorganisms thus obtained, which contain the hybrid plasmid

according to the invention, are separated off from other strains by the selection processes mentioned above.

EXAMPLES

Chromosomal DNA from Bacillus licheniformis DSM 641 or even ATCC 10716 was isolated by Marmur's method (1) with the following modifications: the cells were incubated for 20 minutes in ice in 1 mg lysozyme per ml in 10 mM tris-hydrochloride pH 7.5 containing 25% saccharose before EDTA and sodium dodecylsulfate were added. The extraction with chloroform-isoamylalcohol was carried out only once.

Using a vertical rotor, the DNA was centrifuged for 20 hours at 42,000 r.p.m./20°C in a density gradient (density 1.71 g/cm^3), separated off from other cell constituents and fractionated by dripping out.

Plasmid-DNA from Bacillus subtilis was isolated by Bernhard's method (1), the plasmid-DNA being drawn off by means of a cannula after the centrifuging step in a density gradient.

pBCE 16 (2,4) was used as the vector plasmid for cloning in Bacillus subtilis BR 151 (3).

In order to obtain the gene for subtilisin Carlsberg intact, partial cleavage of the chromosomal DNA of Bacillus licheniformis DSM 641 or ATCC 10716 was carried out with the restriction endonuclease Sau 3A.

Since Sau 3A recognizes a 4-sequence, whereas Bam HI recognizes a 6-sequence, i.e. Sau 3A cuts more frequently, the cleavage sites for incomplete cleavage with Sau 3A are more uniformly distributed than for cleavage of the chromosomal DNA with Bam HI.

Cleavage was carried out, as in all the following cases, under the conditions of the Maniatis (5) manual, except that for the incomplete cleavage the incubation time which would normally be 1 hour was correspondingly

shortened in order to obtain cleavage products comparable in their center of gravity with the vector pBCE 16 (approx. 3 Md). Approx. 1 unit of enzyme was used per 1 µg DNA. The vector pBCE 16 was linearized with the restriction endonuclease Bam HI which allows the same overlapping ends to be formed as Sau 3A.

After incubation, the enzyme was extracted twice with half the volume of phenol, after which Bam HI-cleaved pBCE 16 and Sau 3A-cleaved chromosomal DNA were mixed in a quantitative ratio of 1:10, the mixture was extracted 5 times with the same volume of ether and then incubated for 20 minutes at -70°C with twice the volume of ethanol and the DNA pelletized by centrifuging.

After drying, the DNA was dissolved in the following buffer: 66 mM tris-(hydroxymethyl)-aminomethane, 5 mM $MgCl_2$, 0.3 mM adenosine triphosphate, 1.5 mM dithiothreitol and 0.07 mg/ml beef serum albumin.

The DNA concentration was adjusted to 200 µg/ml and T4-DNA-ligase was added in a concentration of 1 U per µg DNA used. The ligase reaction was carried out for approx. 18 hours at 16°C. The ligation mixture, i.e. the DNA-fragments ligated with T4-DNA-ligase, were then used for the transformation of competent cells of Bacillus subtilis BR 151.

Competent cells of B. subtilis BR 151 were prepared by Laird's method, as described by Cahn and Fox (6).

0.18 ml 0.1 M $MgCl_2$, 0.13 ml 0.05 M $CaCl_2$ and 0.13 ml 100 mM EGTA (ethyleneglycol-bis-2-aminoethylether-N,N,N',N'-tetraacetate) pH 7.3 were added to and mixed (in this order) with 0.82 ml of competent cells, followed after gentle shaking for 5 minutes at 30°C by the addition of 10 µl or 10 µg of the ligation mixture. After slow shaking (60 r.p.m.) for 30 minutes at 30°C, 1 ml of 2 x HGP (10 g peptone from casein, 5 g yeast extract, 5 g NaCl and 5 g glucose/per litre was added.

After shaking for 90 minutes at 160 r.p.m./30°C, the cells were spread with a glass spatula onto calcium-caseinate-agar (modified after Frazier and Rupp - Merck) additionally containing 0.5% casein and 15 µg/ml tetracycline for the purpose of selection of the transformed cells (100 µl per plate).

The plates were incubated for 48 hours at 37°C.

Using subtilisin Carlsberg-specific antibodies purified by chromotography on diethylaminoethylcellulose (DEAE) in combination with a Boehringer/Mannheim "test kit for the immunological detection of specific gene expression in microorganisms", it was possible to detect 6 protease-positive clones among a total of approximately 32,000 transformants. After repeated inoculation, one of these clones proved to be sufficiently stable for further investigations. The detection of s. Carlsberg was carried out as follows:

PVC films were washed in isopropanol for 2 minutes to remove grease and then dried. 15 films were then incubated for 10 minutes in 40 ml 0.2 M sodium carbonate buffer pH 9.2 containing 0.6 ml DEAE-purified subtilisin Carlsberg-specific IgG-fraction for the purpose of coating. The films were transferred without drying to an after-coating bath and left therein for 10 minutes:

40 ml PBS-buffer*
+ 4.8 mg beef globulin
+40 mg beef serum albumin

*) PBS buffer pH 7.5: 0.05 M KH_2PO_4; 0.1 M NaCl

The films were then dried between paper towels.

The IgG-coated films were then placed on the covered agar plates and incubated for 2 hours at room temperature. After removal of the films, the adhering cells were rinsed off with cold tapwater, after which the films were rinsed

on both sides with PBS-buffer tempered to 60°C + 0.1% beef serum albumin. Without drying, the films were transferred to 50 ml of conjugate bath (50 ml PBS buffer containing 0.1% beef serum albumin + 1 µl peroxidase-conjugated IgG against subtilisin Carlsberg) and left therein for 4 hours at room temperature.

The films were then rinsed with cold tapwater and then on both sides with PBS-buffer tempered to 6°C + 0.05% Tween 20 + 0.1% beef serum albumin. After drying between paper towels, the films were incubated on a substrate (Boehringer Gen-Expressionskit(R), Boehringer Mannheim Gm 611) for the coupled peroxidase. The substrate in question is tetramethylbenzidine dissolved in a gelatin base. After incubation for 10 to 30 minutes at room temperature, a blue-green coloration develops in the presence of positive colonies, i.e. in the presence of subtilisin Carlsberg.

The plasmid-DNA of the stable s. Carlsberg-positive clone (pC 50) was isolated as described above.

For characterization, the pC 50-DNA was subjected to cleavage with various restriction enzymes both individually and in combination with Ava I, Bam HI, Bal I, Eco RI, Hpa II, Pst I and Sst I.

Analysis of the cleavage products separated up by vertical agarose gel electrophoresis (7) produced the plasmid map in the accompanying drawing.

Both the Eco RI/Pst I-fragments concerning the cloned fragment and the Eco RI-fragment joining up on the left were inserted into the sequencing vectors pEMBL 8 and pEMBL 9 (8) and the sequence determined by the chain termination dideoxy method (9).

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The claims defining the invention are as follows:

1. Isolated, double-stranded deoxyribonucleic acids consisting of

- the following structural gene which only codes for a polypeptide:

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   10         20         30         40
...GCGCAAA CCGTTCCTTA CGGCATTCCT CTCATTAAAG
CGGACAAAGT GCAGGCTCAA GGCTTTAAGG GAGCGAATGT
AAAAGTAGCC GTCCTGGATA CAGGAATCCA AGCTTCTCAT
CCGGACTTGA ACGTAGTCGG CGGAGGAAGC TTTGTGGCTC
GCGAAGCTTA TAACACCGAC GGCAACGGAC ACGGCACACA
TGTTGCCGGT ACAGTAGCTG CGCTTGACAA TACAACGGGT
GTATTAGCCG TTGCGCCAAG CGTATCCTTG TACGCGGTTA
AAGTACTGAA TTCAAGCGGA AGCGGATCAT ACAGCGGCAT
TGTAAGCGGA ATCGAGTGGG CGACAACAAA CGGCATGGAT
GTTATCAATA TGAGCCTTGG GGGAGCATCA GGCTCGACAG
CGATGAAACA GGCAGTCGAC AATGCATATG CAAGAGGGGT
TGTCGTTGTA GCTGCAGCAG GGAACAGCGG ATCTTCAGGA
AACACGAATA CAATTGGCTA TCCTGCGAAA TACGATTCTG
TCATCGCTGT TGGTGCGGTA GACTCTAACA GCAACAGAGC
TTCATTTTCC AGCGTCGGAG CAGAGCTTGA AGTCATGGCT
CCTGGCGCAG GCGTATACAG CACTTACCCA ACGAACACTT
ATGCAACATT GAACGGAACG TCAATGGCTT CTCCTCATGT
AGCGGGAGCA GCAGCTTTGA TCTTGTCAAA ACATCCGAAC
CTTTCAGCTT CACAAGTCCG CAACCGTCTC TCCAGCACGG
CGACTTATTT GGGAAGCTAA TTCTACTATG GGAAAGGTCT
                                stop
GATCAATGTC GAAGCTGCCG CTCAA TAA....
```

- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that it has the following structure:

- a promoter which is recognized by the RNA-polymerase of microorganisms of the genus Bacillus,
- a ribosome binding site,
- a start codon,
- a leader sequence,
- a stop codon,

DNA-sequences containing up to 400 base pairs without any special expression effect optionally being present at both ends.

2. Hybrid plasmids for microorganisms of the genus Bacillus, characterized in that they contain at least one double-stranded DNA according to Claim 1 in operable form.

3. Hybrid plasmids as claimed in Claim 2, characterized in that they contain a resistance marker and, for the insertion of foreign DNA, at least one enzyme-specific cleavage site, preferably no more than 1 cleavage site being present per restriction enzyme.

4. Hybrid plasmids as claimed in Claim 2 or 3, characterized in that they contain a resistance marker and, for the insertion of foreign DNA, cleavage sites for specific DNA-cleaving enzymes, preferably no more than four and more preferably only one cleavage site being present for each restriction enzyme.

5. Hybrid plasmids as claimed in any one of Claims 2 to 4,

characterized in that they are derived from pBCE 16 and the deoxyribonucleic acids according to Claim 1 contain Bam HI inserted into the cleavage site.

6. A process for selecting microorganism strains containing hybrid plasmids according to Claims 2 to 5, characterized in that

- in a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and
- the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

7. Microorganism strains of the genus bacillus containing hybrid plasmids according to Claims 1 to 6.

8. Microorganism strains as claimed in Claim 7, characterized in that they belong to the species Bacillus subtilis or Bacillus licheniformis.

9. A process for the preparation of subtilisin Carlsberg, characterized in that microorganism strains according to Claims 7 and 8 are cultivated.

10. Isolated, double-stranded deoxyribonucleic acids substantially as hereinbefore described with reference to any one of the Examples.

11. Isolated, double-stranded deoxyribonucleic acids substantially as hereinbefore described with reference to the accompanying drawings.

12. Hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.

13. Hybrid plasmids substantially as hereinbefore described with reference to the accompanying drawings.

14. A process for selecting microorganism strains containing hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.

15. Microorganism strains of the genus bacillus containing hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.

16. A process for the preparation of subtilisin Carlsberg substantially as hereinbefore described with reference to any one of the Examples.

DATED this SIXTEENTH day of OCTOBER 1989
Henkel Kommanditgesellschaft Auf Aktien

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